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14. ABSTRACT Our laboratory has previously shown that two isoforms of Epidermal Growth Factor Receptor family member, HER4: Cyt1 and Cyt2 exhibit opposing effects on mammary epithelial cells *in vitro* and *in vivo*. In our hands, Cyt1 attenuated growth and promoted differentiation, while Cyt2 promoted cell proliferation of mammary epithelial cells. The two isoforms differ by presence of additional 16 amino acids in Cyt1, which introduces a phosphoinositide-3 kinase- and third WW-domain binding motives; however, do not explain the different biological effects of the isoforms. We have focused our studies on a transcriptional regulator and an oncogene, Yap and have shown that Cyt1 preferentially binds Yap as compared to Cyt2. We confirmed these results in COS7 and 293T cells and (using mutational *in vitro* studies and mass spectrometry) identified tyrosines 341 and 394 of Yap as phosphorylation targets by HER4. However, we were unable to replicate these results in mammary epithelial cells, HC11 and MCF7. We also found that HER4 cytoplasmic domain (s80) does not modulate localization of Yap in COS7 cells, although Yap promotes nuclear localization of s80 and these effects are not dependent on isoform nor kinase activity. HER4 expression also did not affect Yap cellular localization in vivo in a transgenic mouse model and human breast carcinoma. Additionally, we evaluated whether HER4 interacts with TEAD, transcriptional factor regulated by Yap, and have found that HER4 forms complex with TEAD; however, this interaction is likely mediated by Yap and we could not find any evidence for HER4-mediated phosphorylation of TEAD. We were also unable to find any transcriptional consequences of HER4 interaction with or tyrosine phosphorylation of Yap. Taken together, our data suggest that HER4:Yap interaction observed in COS7 and 293T cells is nonspecific and due to overexpression of the proteins and either does not occur in mammary epithelial cells or is highly regulated.

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INTRODUCTION

Epidermal growth factor receptor (EGFR) family members are dysregulated in a number of epithelial cell malignancies, including breast cancer[1]. The first three members of the family: EGFR/HER1, HER2 and HER3 promote oncogenic transformation, more proliferative and aggressive cancer[1], and have been successfully targeted by breast cancer treatments. The role of the fourth member of the EGFR family, HER4, in cancer pathogenesis and progression is not as clearly established, but overall expression of HER4 in breast cancer tends to associate with more differentiated histopathological grade, slower proliferating tumors, and favorable patient survival [2-4].

In normal mammary tissue, HER4 is required for proper development and differentiation of the mammary epithelium during pregnancy and lactation [5] and deletion of HER4 in mammary epithelium of mice impairs mammary gland maturation and lactogenesis [5, 6]. *In vitro*, over-expression or activation of HER4 in mammary epithelial cells, normal or cancerous, results in decreased growth, cell cycle arrest, increased apoptosis, and cell differentiation [7-9]. However, in experiments with other cell types including NIH3T3 or COS-7, HER4 over-expression promotes transformation and increased proliferation [10, 11]. Additionally, few studies report a correlation between nuclear localization of HER4 in human breast cancer and worsened patient outcome [11-13]. Thus, additional studies are needed to elucidate the role and signaling mechanisms of HER4 in breast cancer which may significantly aid in development of new targeted therapies or prognostic tests.

The fact that HER4 mRNA can be alternatively spliced into four isoforms (JMa, JMb, Cyt1 and Cyt2 (Fig. 1) may be one possible reason for the conflicting data on the function of HER4 [14, 15]. The JMa isoform contains a TNF-α converting enzyme (TACE) cleavage site in its extracellular region, but which is absent in -JMb isoform. Thus, on ligand-binding, HER4-JMa is cleaved by TACE, leading to shedding of the extracellular domain and undergoes subsequent intramembraneus cleavage by γ-secretase releasing an 80 kDa soluble fragment (s80) that can localize to the nucleus or mitochondria[16], a unique ability among receptor tyrosine kinases. The JMb variant lacks the TACE cleavage site and remains membrane bound. Additional variation in HER4 exists within the cytoplasmic region: the Cyt1 isoform has a 16 amino acid insert that is absent in Cyt2. Human breast cancers express the JMa-Cyt1 and –Cyt2 isoforms[10]; however, most studies of HER4 in breast cancer do not distinguish between these different variants.

Recent studies suggest that Cyt1 and Cyt2 isoforms regulate different cellular processes. HER4 JMa-Cyt2 promotes proliferation of breast (normal and malignant) and other cell lines *in vitro*[11]; whereas Cyt1, through its unique ability to bind and activate phosphoinositide-3 kinase (PI3K), regulates cell survival and chemotaxis[17, 18]. Our laboratory recently compared the effect of these isoforms on growth of mammary epithelial cells and found that expression of s80-Cyt1 slowed cell proliferation and promoted lumen formation in 3D culture consistent with cell

differentiation, while cells expressing s80-Cyt2 proliferated rapidly and failed to differentiate [19]. *In vivo,* induced overexpression of human s80-Cyt1 in mammary epithelium of transgenic mice resulted in decreased proliferation, premature mammary gland maturation and lactogenesis, while induction of s80-Cyt2 resulted in epithelial hyperplasia and disorganization [19]. Additionally, injection of mouse epithelial cells overexpressing Cyt1 isoform of HER4 into a mouse mammary fat pad resulted in formation of tumors that appeared more differentiated and developed slower than control tumors [20]. These findings indicate that Cyt1 and Cyt2 variants activate differentiation and proliferation, respectively; however, the downstream cellular mechanisms regulated by the two isoforms have not been identified.

The Cyt1 and Cyt2 isoforms of HER4 differ only by 16 amino acids, which introduce an overlapping PI3K- and proline-rich, WW domain (PY)- binding motifs in the Cyt1 isoform [14]. The ability of Cyt1, but not Cyt2, to bind and activate PI3K has been demonstrated and shown to regulate cell chemotaxis and survival [17, 18]. New reports confirm that HER4 isoforms exhibit some differential binding preferences as Cyt1 was found to preferentially bind an E3 ligase AIP4/Itch [21], a transcriptional co-activator Yap, and another E3 ligase WWP1 [22]. Interaction of HER4 with these proteins is mediated by the PY motif of s80 and the WW domain of these proteins; thus, presence of an additional PY motif in Cyt1 may explain the biological differences observed between the two isoforms. Functionally, interactions of HER4 with these proteins could regulate HER4 stability [23], signaling (by ubiquitination) [24], as well as ability to modulate transcription [25]. Differences in the interactome of Cyt1 and Cyt2 may explain their opposing effects on cell growth.

One of established binding partners of HER4 is Yap, a protein ubiquitously expressed in mammals, including human and mouse [26, 27]. Yap was initially characterized as a Yes kinase-associated protein [28] and has since been shown to be a transcriptional activator that regulates activity of several transcription factors including RunX1 and 2, p73, and Smad7[29]. However, the primary target of Yap is thought to be the family of TEF/TEAD transcription factors, shown by Zhao *et al.* to be required for Yap-dependent regulation of cell proliferation and transformation[30].

Yap is negatively regulated by the Hippo/Warts pathway[31], which relays signals from the extracellular environment into the nucleus and through Yap-mediated regulation of cell proliferation, differentiation, and apoptosis determines organ size during development[29]. Recently the Hippo pathway has also been shown to mediate the cell-cell contact inhibition[31].

Yap is also recognized as an oncogene as A) it is encoded on human chromosome 11q22, a region often amplified in human cancers, as well as mouse models of liver[32] and breast cancer[33], B) over-expression of Yap in the non-transformed mammary epithelial cell line (MCF10A) increases cell migration, epithelial-to-mesenchymal transition, growth factor-independent proliferation, and anchorage independent growth[33], and C) conditional over-expression of Yap in mouse liver leads to hepatomegaly and development of liver tumors[34].

BODY

Published data establish that HER4 and Yap are binding partners and our preliminary data indicate that HER4 Cyt1 exhibits enhanced ability to bind Yap as compared to Cyt2. We hypothesized that HER4 isoforms may distinctly regulate Yap activity through their differential ability to interact with Yap, which may explain their opposing effects on mammary epithelial cells. We thus designed this study to further compare the interaction of HER4 isoforms, Cyt1 and Cyt2 with Yap (Aim 1), and evaluate the effects of this interaction on Yap localization *in vitro* and *in vivo* (Aim 2) and function (Aim 3). We summarize here the research results obtained during the two years of the Fellowship.

Aim 1: To characterize the interaction of HER4 s80 –Cyt1 and –Cyt2 with Yap. We aimed to confirm the differential ability of HER4 Cyt1 and Cyt2 isoforms to phosphorylate Yap in mammary epithelial cell lines and to determine which Yap tyrosine(s) are targeted for phosphorylation by the HER4 isoforms.

Subtask 1a: Generation of Yap mutants.

Using Mutagenesis Kit (Pierce) and manufacturer's software to design primers, we generated four constructs encoding HA-tagged Yap1 harboring Tyr-to-Phe substitution for each of the four tyrosines present in Yap1 (Fig. 2). The mutations were introducedinto Yap1 instead of Yap2, as originally proposed, due to the fact that Yap1 is the predominant isoform expressed in human [26]. We also realized that for mapping of the phosphorylation site, it will be adequate to generate mutants with single mutations, instead of combinations of mutated tyrosines, which will also decrease the possibility of any effects on protein structure due to multiple amino acid substitutions. To simplify annotation, the generated constructs were designated Y1F, Y2F, Y3F and Y4F, where each abbreviation corresponds to Y188F, Y341F, Y357F, and Y394F, respectively.

Subtask 1b. Identification of Yap tyrosine(s) targeted by s80-Cyt1 and –Cyt2 for phosphorylation in COS7 and 293T cells.

Our preliminary data indicated that HER4 binds Yap and that Cyt1 isoform binds Yap with higher affinity, as compared to Cyt2, likely contributing to enhanced phosphorylation of Yap (Fig. 3a). We confirmed these findings in two different cell lines, COS7 and 293T cells and identified the Yap tyrosine targeted for phosphorylation.

To accomplish this task, HA-tagged Yap1 or the Tyr-mutants were transiently co-expressed with Flag-tagged HER4-s80-Cyt1, -Cyt2 or kinase dead (KD) mutants in COS7 or 293T cells using Fugene (Roche) and following manufacturer's protocol. Cells were lysed 48h after transfection and immunoblotting analysis performed either directly on cell lysate or on immunoprecipitated fraction, as indicated.

We first evaluated the ability of other EGFR family members to bind Yap and found that only HER4 co-immunoprecipitates with Yap (Fig. 3b). In further experiments, we confirmed the preliminary findings that in COS7 and 293T cells Cyt1 preferentially binds Yap as compared to Cyt2 (Fig. 3c and d). Our data also indicate that HER4 interaction with Yap does not require kinase activity (Fig.3c and d). Subsequently, using the Yap1 mutants generated in Subtask 1a, we determined that HER4 targets the last tyrosine within the transactivation domain of Yap1 for phosphorylation (Y394, Fig. 4a). These findings were confirmed by mass spectrometry (MS) with help of the University of North Carolina Michael Hooker Proteomics Center (Fig. 4b and Table 1). The MS studies also indicated high probability of HER4 phosphorylating tyrosine 341 of Yap1, which was confirmed on manual inspection of the spectrum. However, this was not detected by immunoblot when HER4 and Yap1-Y2F were co-expressed (Fig. 4a and c) suggesting that phosphorylation of Y341 maybe be dependent on and secondary to Y394 phosphorylation. Thus, in Yap1 lacking Y394, both phosphorylation sites would be affected and no phosphorylation would be detected, while in Y341 mutation, only one site of phosphorylation would be lost, which might not be discernible on an immunoblot. Additionally, in some experiments we observed a decrease in tyrosine phosphorylation of Yap when tyrosine 188 was mutated (compare Fig. 4c vs. 4a); however, we attribute this finding to loss of binding between HER4 s80 and Yap which is known to be mediated by the WW domain of Yap and is dependent on the tyrosine [27]. Correspondingly, we observed less or no HER4-s80 co-immunoprecipitate with Yap1 Y1F (Fig. 4a and c). Subsequently, we compared whether HER4 isoforms target the same tyrosine(s) of Yap1 for phosphorylation and indeed found that both Cyt1 and Cyt2 phosphorylate the same tyrosine(s) (Fig. 4c).

<u>Subtask 1c. Confirmation of s80-Cyt1 and –Cyt2 binding with and phosphorylation of Yap in mammary epithelial cells (HC11 and MCF7).</u>

We evaluated whether HER4 binds Yap in mammary epithelial cells (HC11 and MCF7) following a similar experimental protocol as that used for COS7 and HEK293 cells. However, we found that transient transfection of HC11 or MCF7 cells using standard Fugene (Roche) protocol results in low levels of protein expression. Attempts to optimize Yap and HER4-s80 expression and detection in these cells (using alternate expression methods including electroporation, reverse transfection and retroviral expression constructs, modified lysis buffers and sonication) did result in higher levels of expression and better detection (Fig. 5 a-c); however, we were not able to reproducibly detect Yap:HER4-s80 interaction nor Yap tyrosine phosphorylation. Despite strong s80 expression, interaction of s80:Yap was detected in only a few experiments and detection of tyrosine phosphorylation of Yap was only seen after 5 min pretreatment of samples with pervanadate, a potent and promiscuous inhibitor of all tyrosine phosphatases (Fig. 5c).

Similar difficulties were encountered while investigating Yap and HER4-s80 interaction in MCF7 cells. Initial attempts at transient transfection yielded low levels of expression; although, were improved greatly by employing reverse transfection protocol (Fig. 6). However, despite

good expression levels of both Yap and HER4-s80, we were unable to detect any interaction between these two proteins, nor tyrosine phosphorylation of Yap.

In hopes to overcome these technical difficulties, we developed additional HC11 and MCF7 cell lines expressing Flag-tagged HER4-s80-Cyt1, –Cyt2, or kinase dead (KD) variants under control of doxycycline-inducible promoter (RetroX retroviral TetOn system, Clonetech). After double retroviral infection with retrovirus and selection for minimum of two weeks, cell lines exhibited strong expression of Flag-tagged HER4-s80 isoforms only in presence of doxycycline, as early as 2-4h after induction (Fig. 7a and data not shown). However, despite good detection of endogenous Yap (Fig. 7b) and s80 expression (Fig. 7a), we were unable to demonstrate co-immunoprecipitation of the two proteins in these mammary epithelial cell lines.

In a final attempt to detect interaction of Yap and the cytoplasmic domain of HER4 isoforms, we turned to a novel Proximity Ligation Assay developed by Olink Biosciences. This assay employs in-cell antibody-mediated detection of protein of interest and nucleic acid amplifation to detect protein interactions with much greater sensitivity than co-immunoprecipitation while at the same time preserving cell structure[35]. Briefly, cells grown on slides are fixed, permeabilized and interrogated for proteins of interest with primary antibodies raised in two different species. Secondary antibodies coupled to oligonucleotides are used for detection, and if the oligonucleotides are within 40 nm of each other, they are ligated, amplified and labeled with fluorophore which enables specific detection of protein interaction by fluorescent or confocal microscopy. We applied this protocol to HC11 TetOn cells grown on slides in presence and absence of doxycycline (to induce HER4 s80 expression) to detect interaction of s80-Cyt1 or –Cyt2 with endogenous Yap. As expected, there was no signal detected in control cells indicating lack of interaction (Fig. 8, Panel 1); however, faint signal was detected in both Cyt1 and Cyt2 cells in absence of doxycycline. Signal was also detected in all cells exposed to doxycycline for 24h (including parental cells; Fig. 8, Panel 2), bringing into question specificity of this assay. It is possible that the PL assay is so sensitive that it uncovers leaky expression of HER4-s80 cells in absence of doxycycline, which might be below the level of detection on immunoblots. However, presence of the signal in parental cells brings into question reliability and sensitivity of this assay and extensive evaluation would be needed to confirm these results.

Together, these results indicate that HER4-s80 interaction with Yap may be cell type specific and does not occur in mammary epithelial cells or is prevented due to compartmentalization or some regulatory mechanism(s) that are able to compensate for protein overexpression. COS7 and 293T cells either lack these mechanisms due to cell immortalization/transformation or are not able to compensate for very high expression of both Yap and HER4-s80, leading to artificial protein interactions. It is important to note, that in mammary epithelial cells *in vivo*, HER4 expression is maintained at very low levels (even in cells overexpressing HER4-s80 enrichment by immunoprecipitation is required for detection by immunoblot). Alternatively, the HER4:Yap interaction might be only occurring in the nucleus of the mammary epithelial cells and the interacting proteins are retained within the insoluble fraction during cell lysis. We examined Yap

and HER4-s80 cellular localization using cellular fractionation kit (Pierce) and could not detect these proteins in the nucleus (data not shown). Requirement of the nuclear fractionation method for high salt buffer prevented us from performing co-immunoprecipitation experiments. Lastly, sonication, which greatly improves protein detection in HC11 and MCF7 cell lysates, may be causing disruption of the protein complexes and nonspecific protein aggregation[36] and thus prevent detection of HER4-s80:Yap interaction. We have tested different lysis buffers, but were unable to detect transfected proteins as well as when sonication was used.

Aim 2. To determine whether HER4 isoforms differentially regulate Yap localization. We will explore the role of HER4 s80 as a potential Yap nuclear transporter and compare the ability of Cyt1 and Cyt2 to regulate Yap localization in three different models of breast cancer.

Subtask 2a. Evaluation of cellular localization of s80-Cyt1, -Cyt2 and Yap.

We examined Yap and HER4-s80 localization in COS7 cells and found that Yap influences the localization of HER4 cytoplasmic domain (Fig. 9). To complete this analysis, COS7 cells were co-transfected with HA-tagged Yap and Flag-tagged HER4-s80-Cyt1, -Cyt2 or KD and 24h later plated on chamber slides. After 24h, cells were fixed and stained using anti-HA and anti-Flag antibodies coupled to Alexa-647 or -488, respectively, and after mounting with DAPI-containing media (to counterstain the nuclei), analyzed by confocal microscopy with assistance of the Microscopy Services Laboratory at the University of North Carolina. Our data indicate that Yap expressed alone distributes throughout the cell, both in the cytoplasm and the nucleus, with some focal localization in the cytoplasm (Fig. 9, Panel 2, C). Both HER4-s80 isoforms also distributed throughout the cell, both in the cytoplasm and nucleus (Fig. 9, Panel 1, F, J, N, R). We did not observe any differences in localization of Cyt1 and Cyt2 isoforms, nor the KD mutants, which indicates that HER4 does not depend on its kinase activity for nuclear localization. These findings are in direct contrast with published data from our laboratory [16] and might be due to the fact that we employed COS7 cells for these analyses, while the published data was generated in HC11 cells. As discussed above, HER4 localization and signaling may be regulated in cell-specific manner. Alternatively, gross overexpression of HER4 in COS7 cells could result in protein accumulation in the nucleus.

However, when Yap and HER4-s80 were co-expressed, s80 localized to the nucleus in very distinct focal regions and co-localized with Yap within those regions (Fig.9E-T, Panel 2). Interestingly, Yap localization did not change significantly, and Yap was still observed in both cytoplasm and nucleus, although exhibited more focal localization within the nucleus (Fig.9, Panel 2, C vs. G, K, O, S). These data indicate that Yap localization to the nucleus is independent of HER4 cytoplasmic domain, but that Yap promotes s80 localization to the nucleus and to specific focal regions. These results further confirm that HER4-s80 bind Yap in COS7 cells, as reported above in Subtask 1b.

Due to difficulties with detection of HER4:Yap interaction in mammary epithelial cells (described above), we were unable to confirm these results in mammary epithelial cells.

Subtask 2b: Characterization of the effect of s80-Cyt1 and –Cyt2 expression on Yap localization in mouse mammary glands.

To evaluate Yap localization in mammary glands of transgenic mice, we stained paraffin-embedded mammary glands with anti-Yap antibody (Cell Signaling) following an established immunohistochemistry protocol. We performed the analysis on all available samples that were generated previously in our lab (see Muraoka-Cook *et al.* 2009) and approved under IACUC protocol 06-178 on 06/15/2006.

In our analysis we included mammary glands from WT mice (controls) and transgenic mice expressing s80-Cyt1, s80-Cyt2, or degradation-resistant Cyt1 (db) that were exposed to doxycycline (to induce transgene expression) for either 4 days or one year (Table 2).

Yap was found to localize to both cytoplasm and the nucleus of mammary epithelial cells in the control mice (Fig. 10). We did not observe any differences in Yap localization between tissue from WT, Cyt1^{+}/db[+] or Cyt2^{+} mice, which corresponds to our findings from the *in vitro* localization experiments reported above. Differences in staining intensity are due to batch differences.

Subtask 2c. Evaluation of the relationship between HER4 and Yap nuclear localization in estrogen receptor positive human breast cancer samples.

We performed a pilot study on 10 estrogen receptor positive human breast carcinomas and matching normal controls. The samples were stained for HER4 and Yap using established immunohistochemistry protocol. Within the normal samples examined, we observed HER4 expression in mammary epithelium as well as weaker staining in the stroma. HER4 predominantly localized to the cytoplasm in the normal mammary epithelium, with a few cells exhibiting nuclear localization (Fig. 11A-C). In the carcinoma tissue, we observed slight decrease in intensity of cytoplasmic staining for HER4 in nine of ten samples, but only two samples with increased nuclear stain (Fig. 11D-F).

Yap was observed only in the mammary epithelium in samples obtained from normal tissue, where it localized primarily in the cytoplasm (Fig. 11G-I). In the carcinoma samples, Yap cytoplasmic expression decreased in 7 out of ten samples, however there were no changes in nuclear expression in those samples (Fig. 11J-L). We observed increase in Yap cytoplasmic expression in only one carcinoma sample as compared to its matching control.

In eight samples out of ten tested we observed changes in both HER4 and Yap expression; however there was no particular trend or directionality that would indicate any relationship in localization or expression of the two proteins. As we have been unable to detect any trends in

Yap or HER4 localization in the carcinoma samples as compared to normal matching tissue, we halted further analysis.

Aim 3: To evaluate the functional consequences of HER4 isoform interaction with Yap. We investigated whether binding and phosphorylation of Yap by HER4 isoforms modulate the ability of Yap to regulate TEF/TEAD-, RunX2-, and p73-dependent transcription. We also examined whether HER4 s80–Cyt1 and –Cyt2 interact with the Yap:transcription factor complex, specifically Yap:TEF/TEAD, and evaluated the ability of HER4 isoforms to directly bind and phosphorylate TEF/TEAD.

Subtask 3a. Evaluation of the ability of HER4 isoforms to modulate transcriptional regulatory activity of Yap.

To evaluate the ability of HER4 isoforms, Cyt1 and Cyt2, to modulate the transcriptional activity of Yap, we co-expressed Yap, TEF/TEAD and HER4 isoforms in COS7 or 293T cells. Concurrently, cells were transfected with a CTGF-promoter driven luciferase construct, as CTGF is an established TEF/TEAD-dependent gene [30].

In 293T cells, Yap increased expression of the luciferase reporter, as compared to expression of the reporter alone. However, co-expression of HER4 isoforms did not modulate Yap activity as evidenced by lack of change in reporter expression under these conditions (Fig. 12A).

Similarily, in COS7 cells, Yap increased reporter expression 5-10 fold and this effect was further augmented by co-expression of the TEF/TEAD. HER4 isoforms did not modulate the observed effect; although, there was a non-significant trend for increased reporter expression in presence of Cyt1 and decreased expression in presence of Cyt2 (Fig. 12B). These experiments were also conducted in HC11 mammary epithelial cell line, with similar results (data not shown).

We additionally employed quantitative PCR to test the ability of HER4 isoforms to modulate Yap transcriptional activity. We used the endogenous CTGF, a target of TEF/TEAD [30], and Bax, p73 target [37, 38], as indicators of changes in Yap transcriptional activity. The data show, that despite adequate expression of Yap and HER4 isoforms in MCF7 mammary epithelial cells, the levels of CTGF and Bax remained unchanged (Fig. 12C).

Subtask 3b. Confirmation of the role of HER4-dependent tyrosine phosphorylation of Yap in modulation of Yap transcriptional activity.

Due to lack of observed effect in subaim 3a, we performed a limited study in MCF7 mammary epithelial cells to evaluate whether tyrosine phosphorylation resisitant Yap, generated in subaim 1a, exhibited change in transcriptional activity as compared to wild type Yap.

MCF7 cells were transfected with Yap or Yap Y4F mutant. Only Y4F mutant was selected since this was the primary tyrosine found phosphorylated on Yap by HER4, as described in subaim 1b. We employed the quantitative PCR to measure the expression levels of two Yap-regulated genes:

CTGF (via TEF/TEAD transcription factor) and Bax (via p73 transcription factor). Due to previous difficulties in expressing constructs in MCF7 cells, the expression levels of WT and mutant Yap, and HER4 isoforms were confirmed by immunoblot, and were found adequate (Fig. 13A). However, despite good expression, we did not observe any effect of Yap or HER4 on the expression levels of the reporter genes (Fig. 13B). Due to lack of effect, further studies proposed for this subaim were not pursued.

Subtask 3c. Determination whether HER4 isoforms directly bind and tyrosine phosphorylate TEF/TEAD transcription factor.

To evaluate whether HER4 isoforms, Cyt1 and Cyt2, bind the TEF/TEAD transcription factor directly, COS7 cells were transiently transfected with Myc-tagged TEF/TEAD, HA-tagged Yap, and Flag-tagged HER4 isoforms. Lysates were generated 48h after transfection and immunoprecipitated for TEF/TEAD and associated proteins. The enriched lysates were then interrogated via immunoblots to detect any association between the HER4, Yap and TEF/TEAD proteins (Fig. 14A).

Our data indicate that both Cyt1 and Cyt2 associate with TEF/TEAD and that this interaction may be stabilized by Yap. We also observed that TEF/TEAD is tyrosine phosphorylated in presence of HER4, regardless of Yap co-expression.

Interestingly, ectopic co-expression of Cyt1 and TEF/TEAD in COS7 cells resulted in decreased levels of these two proteins. However, inhibition of the proteasomal (with MG132) and lysosomal (with Bafilomycin A) degradation pathways did not reverse the observed effect (Fig. 14B), indicating possible changes in transcription.

KEY RESEARCH ACCOMPLISHMENTS:

- Generated constructs encoding HA-tagged human Yap1 (wild type) and Yap1 harboring Tyr-to-Phe mutations for each of its tyrosines (Y188F, Y341F, Y357F, and Y394F)
- Confirmed preliminary data in COS7 and 293Tcells supporting our findings that Yap is preferentially bound by HER4-s80-Cyt1 as compared to –Cyt2
- Identified Tyr 341 and Tyr394 as Yap1 tyrosines that are phosphorylated by HER4-s80 (both Cyt1 and Cyt2), with the Tyr394 being the major target
- Found that HER4-s80 either does not bind and phosphorylate Yap in mammary epithelial cells, or the interaction is subject to very stringent regulation
- Found that Yap promotes nuclear and focal localization of both HER4-s80 isoforms, which is independent of HER4 kinase activity and that HER4-s80 co-localizes with Yap. However, HER4-s80 does not modulate Yap cellular localization.
- Found that HER4-s80 does not affect Yap cellular localization in murine mammary gland *in vivo*.
- Did not find any relationship between HER4 and Yap expression and localization in human breast carcinoma as compared to normal tissue.
- Did not find any evidence for the ability of HER4 isoforms and Yap tyrosine phosphorylation to modulate transcriptional activity of Yap
- Found that HER4 may form a complex with and tyrosine phosphorylate TEF/TEAD transcription factor and that Yap stabilizes this interaction.

REPORTABLE OUTCOMES:

Misior A, Earp HS. Mediators of HER4 Cyt1 and Cyt2 isoform signaling: role of WW domain proteins. Postdoctoral Research Symposium. Abstract for an oral presentation. University of North Carolina at Chapel Hill, Chapel Hill, NC. October 2009.

Completed Cancer Pathology course (PATH725) offered by the UNC Cancer Cell Biology Training Program in May 2010

Misior A, Feng S, Earp HS. HER4 isoforms Cyt1 and Cyt2 differentially interact with Hippo pathway effectors Yap and TEAD. Postdoctoral Research Symposium. Abstract for a poster. University of North Carolina at Chapel Hill, Chapel Hill, NC. October 2010.

Misior A, Feng S, Hashmonay G, Earp HS. HER4 isoforms Cyt1 and Cyt2 differentially interact with Hippo pathway effector Yap. Abstract for a poster. Department of Defense Era of Hope Conference, Orlando, FL. August 2-5, 2011.

CONCLUSION

The data collected over the past two years indicate that HER4 is the only EGFR family receptor that binds Yap and that, in COS7 and 293T cells, the cytoplasmic domain of HER4 binds and phosphorylates Yap. Cyt1 isoform preferentially binds Yap, as compared to Cyt2 and both Cyt1 and Cyt2, phosphorylate Yap on Tyr341 and Tyr394, localized within the transactivation domain of Yap1.

This is the first report of Yap phosphorylation by HER4. Previously, Yap has been shown to be phosphorylated by c-Abl, which targets Tyr357, also localized within the transactivation domain of Yap (in our notation, annotated as Y3F, see Fig.2)[38]. This modification increased stability of Yap, promoted its binding with transcriptional factor p73, and switched p73-driven gene expression from growth arrest to proapoptotic genes. In another report, Zaidi *et al.* show that Yap is phosphorylated by Src, although the site of phosphorylation was not identified, which promoted binding of Yap with transcriptional factor RunX2, and inhibited expression of RunX2-dependent genes [37]. In both of these instances tyrosine phosphorylation of Yap modulated gene expression of Yap-regulated transcription factors; however, we were unable to detect any functional consequence of Yap tyrosine phosphorylation by either HER4 isoform.

Similarly, we were unable to conclusively confirm that HER4 and Yap interact in mammary epithelial cells despite achieving adequate expression of both proteins and using a variety of techniques. It is possible that HER4 and Yap interaction is cell-type specific and localization of HER4 and/or Yap might be highly regulated/compartmentalized in mammary epithelial cells. HER4 is required for differentiation of mammary epithelial cells[5], thus it may be highly regulated in these cells. Additionally, the two models we selected for the studies, the HC11 and MCF7 cells, despite being cell lines still maintain "normal" mammary cell biology, evidenced by their ability to differentiate when exposed to lactogenic hormones (HC11 cells)[39] and expression of estrogen receptor (MCF7 cells)[40]. We evaluated interaction of HER4 and Yap in these cells using a variety of methods (immunoprecipitation and immunoblotting, confocal microscopy and proximity ligation assay), as well as models (transient and stable transfection, inducible expression). However, all without success, indicating strongly that HER4 and Yap do not interact in these mammary epithelial cell lines.

We also found some limited evidence that tyrosine phosphorylation of Yap is enhanced in HER4-s80-Cyt1-expressing mammary epithelial cells. However, we were only able to detect the phosphorylation when cells were pretreated with pervanadate, a potent and promiscuous inhibitor of tyrosine phosphatases. Interestingly, we could detect HER4-s80 tyrosine phosphorylation also only after block of phosphatase activity, while in other cells studied (COS7 and 293T) the phosphorylation was readily detected. These results suggest strict regulation of HER4-s80 in mammary epithelial cells.

Studies conducted in aim 2 indicate that HER4-s80 isoforms do not modulate Yap localization in COS7 cells; rather, Yap drives nuclear localization of HER4 to specific foci. The distribution of HER4-s80 did not differ between the Cyt1 and Cyt2 isoforms and was not dependent on kinase activity. These findings are in direct contrast to published findings from our laboratory[16], where nuclear localization of HER4-s80 was shown to require active kinase. The published studies were conducted in HC11 mammary epithelial cells, whereas our current studies were carried out in COS7 cells, and might be another evidence for cell-type specific regulation of HER4 signaling.

We also assessed the effect of HER4 isoform expression on endogenous Yap localization *in vivo*, utilizing a transgenic mouse model expressing human HER4-s80-Cyt1 or –Cyt2 in the mammary gland under control of doxycycline-responsive promoter[41]. We have analyzed all samples available from previous studies in our laboratory (no new mice were generated for this study), and found no differences in Yap cellular localization regardless of transgene expressed (Cyt1/Cyt1^{db} or Cyt2).

Similarly, we were unable to find any relationship between HER4 and Yap expression in human ER$^+$ breast carcinoma samples. Published data do not agree on expression and localization of HER4 in breast carcinoma: some find less than 50%, while others report that 70% of breast cancers express HER4[42]. In the most recent and most comprehensive analysis of HER4 expression in invasive breast carcinoma, Thor *et al.*[13] analyzed 923 samples and found HER4 expression in 68% of patients. The authors also reported that HER4 localized to the cytoplasm in 63% of samples, to the nucleus in 23%, and to both in 18%. In our limited analysis, we predominantly observed HER4 positivity in cytoplasm and only few cells exhibited nuclear localization. We did not observe significant differences in HER4 localization between normal tissue and carcinoma samples.

Yap is reported to be expressed primarily in mammary epithelial cells and myoepithelial cells, both in cytoplasmic and nuclear compartments[43] and we observed similar localization. Similarly, we did not observe any changes in Yap localization in breast carcinoma as compared to normal tissue in our pilot study of ten patient samples. Our results are supported by a recent report by the Tang group [39], who analyzed Yap expression in breast cancer samples from 94 patients using tissue microarray and found no significant relationship between Yap expression and clinical variables.

Aim 3 studies, completed during the second and final year of the fellowship, supported our results from Aims 1 and 2, and indicated lack of functional effects of HER4 interaction with Yap in all cells studied (COS7, 293T, HC11 and MCF7). We did find evidence for formation of HER4:TEF/TEAD complex, and its stabilization by Yap. However, due to expression of endogenous Yap in these cells, we cannot conclude that the two proteins bind directly without Yap participation in the complex. In summary, our data lead us to conclude that the observed binding of HER4 isoforms Cyt1 and Cyt2 and Yap in COS7 and 293T cells is likely a result of

gross overexpression of these proteins and does not occur in mammary epithelial cells or is prevented by some regulatory mechanism (i.e. compartmentalization) that are able to compensate for protein overexpression. COS7 and 293T cells either lack these mechanisms or are not able to compensate for very high expression of both Yap and HER4-s80, leading to artificial protein interactions.

Even though the data reject our hypothesis, they provide valuable information about HER4-dependent mechanisms and cell type specific effects, as well as information about the models we should employ in future studies. We will continue characterizing HER4 signaling, specifically differences between the Cyt1 and Cyt2 isoforms in mammary epithelial cells, but will focus on proteomic and genomic approaches to identify novel mediators and will work towards better understanding of HER4 role in mammary gland development and breast cancer pathogenesis.

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APPENDIX I: REPORTABLE OUTCOMES

Misior A, Earp HS. Mediators of HER4 Cyt1 and Cyt2 isoform signaling: role of WW domain proteins. Postdoctoral Research Symposium. Abstract for an oral presentation. University of North Carolina at Chapel Hill, Chapel Hill, NC. October 2009.

Overexpression of epidermal growth factor receptor (EGFR), HER2, and HER3, first three members of the EGFR family correlates with more aggressive and metastatic breast cancer. However, the relationship between expression of the fourth receptor in the family, HER4, and clinical cancer progression is more complex. Often, HER4 expression corresponds to more differentiated, therapy-responsive tumor and lower rate of reoccurrence.

Due to alternate RNA splicing, HER4 can be expressed as four different isoforms that differ in their cellular localization and signaling capabilities. Human breast cancers express two isoforms, JMa -Cyt1 and -Cyt2, which differ only by presence of a 16 amino acid (aa) insert within the cytoplasmic domain of Cyt1, but not Cyt2. This sequence contains putative binding motifs for phosphoinositide-3-kinase (PI3K) and WW domain proteins (WWDPs). Studies have confirmed that only Cyt1 is capable of activating PI3K, but differences in the ability of the two isoforms to interact with WWDPs have not been explored.

We recently demonstrated that expression of the soluble cytoplasmic domain (s80) -Cyt1 or -Cyt2 *in vitro* and *in vivo* results in strikingly different phenotypes. In 3D cell culture and in transgenic mouse models, expression of s80-Cyt1 corresponded with decreased cell proliferation and increased differentiation, while expression of s80-Cyt2 enhanced cell proliferation and tissue disorganization. These data suggest that Cyt1 and Cyt2 variants activate differentiation and proliferation, respectively. Additionally, we compared the ability of Cyt1 and Cyt2 to bind WWDPs, and observed that Cyt1 and Cyt2 interact with some WWDPs with similar affinities, but they preferentially associate with others. One of the WWDPs found to differentially bind HER4 isoforms is transcriptional regulator and a putative oncogene Yap. Our studies aim to further characterize interaction of Yap with HER4 isoforms and its functional consequences.

Misior A, Feng S, Earp HS. HER4 isoforms Cyt1 and Cyt2 differentially interact with Hippo pathway effectors Yap and TEAD. Postdoctoral Research Symposium. Abstract for a poster. University of North Carolina at Chapel Hill, Chapel Hill, NC. October 2010.

In contrast to the first three members of the epidermal growth factor receptor (EGFR) family (EGFR, HER2, and HER3), HER4 appears to have a protective role in breast cancer. Often, its expression corresponds to more differentiated, therapy-responsive tumor and lower rate of recurrence.

HER4 can be expressed as four different isoforms that differ in their cellular localization and signaling capabilities. Human breast cancers express two isoforms, JMa -Cyt1 and -Cyt2, which promote strikingly different phenotypes *in vitro* and *in vivo*. Expression of the cytoplasmic domain of HER4-Cyt1 corresponds with decreased cell proliferation and increased differentiation, while expression of -Cyt2 enhances cell proliferation and tissue disorganization. These data suggest that Cyt1 and Cyt2 variants of HER4 activate differentiation and proliferation, respectively; however, the molecular mechanisms responsible for these effects have not been elucidated.

It has been established that upon ligand binding HER4-JMa cytoplasmic domain is released from the membrane and translocates to the nucleus. Using cell culture and biochemical approaches, we investigated the role of HER4-JMa-Cyt1 and –Cyt2 in the nucleus and identified two transcriptional regulators, Yap and TEAD, as novel targets of HER4 tyrosine kinase. In addition, our data indicate that HER4-Cyt1 isoform preferentially interacts with and phosphorylates these transcriptional regulators. As Yap and TEAD are targets of the Hippo pathway, responsible for regulation of cell growth through contact inhibition, their regulation by Cyt1 may mediate the pro-differentiation effect of HER4-Cyt1 on mammary epithelium. Our findings identify novel mediators of HER4 signaling leading to a better understanding of the differential effects of Cyt1 and Cyt2 isoforms, and revealing new potential targets for development of breast cancer therapies.

Misior A, Feng S, Hashmonay G, Earp HS. HER4 isoforms Cyt1 and Cyt2 differentially interact with Hippo pathway effector Yap. Abstract for a poster. Department of Defense Era of Hope Conference, Orlando, FL. August 2-5, 2011.

Overexpression of the first three members of the epidermal growth factor receptor (EGFR) family: EGFR HER2, and HER3, correlates with more aggressive and metastatic breast cancer. However, the relationship between expression of the fourth receptor in the family, HER4, and clinical cancer progression is more complex. Often, HER4 expression corresponds to more differentiated, therapy-responsive tumor and lower rate of recurrence.
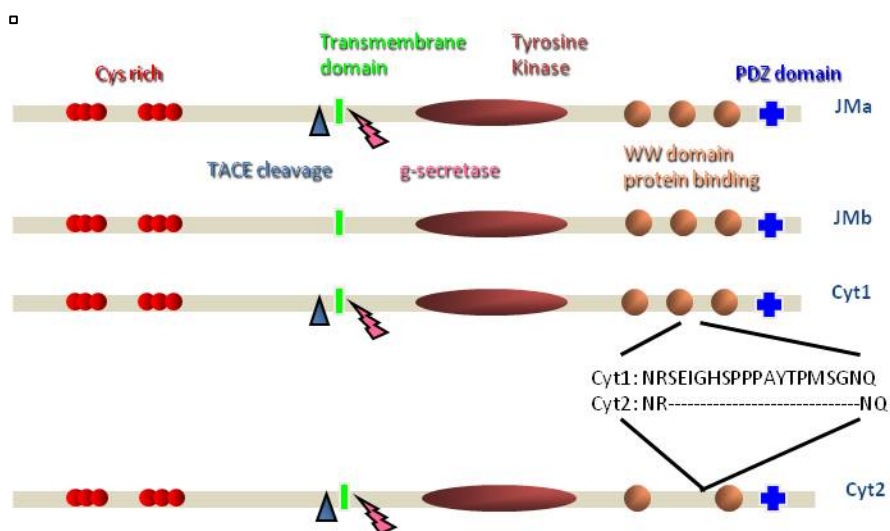
Due to alternate RNA splicing, HER4 can be expressed as four different isoforms that differ in their cellular localization and signaling capabilities and two isoforms, JMa -Cyt1 and -Cyt2 have been identified in the human breast cancers. Our laboratory has demonstrated that expression of the soluble cytoplasmic domain (s80) of Cyt1 or Cyt2 *in vitro* and *in vivo* results in strikingly different phenotypes: overexpression of s80-Cyt1 corresponds with decreased cell proliferation and increased differentiation, while expression of s80-Cyt2 enhances cell proliferation and tissue disorganization. These data suggest that Cyt1 and Cyt2 activate differentiation and proliferation, respectively.

The HER4 Cyt1 and Cyt2 isoforms differ only by presence of an additional 16 amino acid insert within the cytoplasmic domain of Cyt1, which contains binding motifs for phosphoinositide-3-kinase and WW domain proteins (WWDPs). Using WW domain protein arrays, we identified Yes-associated protein (YAP) as one of the proteins bound by the 16 amino acid unique to Cyt1. YAP is a transcriptional modulator regulated by the HIPPO pathway responsible for cell-cell contact inhibition and control of organ size. Additionally, YAP has been recently identified as an oncogene. Our preliminary studies found that Yap binds both HER4 isoforms, but preferentially associates with s80-Cyt1. **Based on these data, we hypothesized that Cyt1 and Cyt2 differentially interact with and regulate YAP, which may contribute to their opposing effects on mammary epithelial cell proliferation and differentiation.** Consequently, employing *in vitro* co-expression strategies and immunoblotting, we compared interaction of s80-Cyt1 and -Cyt2 with, and their ability to phosphorylate, YAP, and regulate YAP's activity. Our studies confirm that YAP is preferentially bound by HER4-Cyt1 and identify YAP as a novel target of HER4 tyrosine kinase. Further, using mutagenesis and proteomic approaches, we identified Y394 as the tyrosine targeted by HER4. Further studies evaluate the functional consequences of HER4-Cyt1 and -Cyt2 interaction with and tyrosine phosphorylation of YAP. Regulation of YAP activity may mediate the pro-differentiation effects of HER4-Cyt1 on mammary epithelium. Our findings identify novel mediators of HER4 signaling leading to a better understanding of the differential effects of Cyt1 and Cyt2 isoforms, and revealing new potential targets for development of breast cancer prognostic markers and therapies.

APPENDIX II: PERSONNEL SUPPORTED BY THE FELLOWSHIP

Anna M. Misior, PhD

Figure 1. HER4 isoforms. Due to alternative mRNA splicing, HER4 exists as four isoforms: JMa contains a TACE cleavage site in the transmembrane region, which is absent in JMb, while Cyt1 contains additional 16 amino acids within its cytoplasmic domain. The TACE cleavage site allows for JMa to be cleaved by TACE and subsequently be processed by γ-secretase to release an 80 kDa cytoplasmic domain (s80), which has been shown to translocate to the nucleus and mitochondria. Extra 16 amino acids present in Cyt1 contain a PI3K and WW domain binding motives.
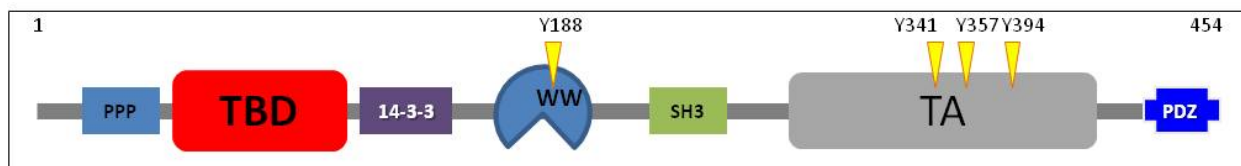

Figure 2. Domain structure of Yap. Diagram depicting domain structure of predominant human Yap isoform (Yap1). Yap contains a proline rich region (PPP), TEF/TEAD binding domain (TBD), 14-3-3 protein binding motif (14-3-3), WW domain (WW), SH3 binding motif (SH3), transactivation domain (TA), and a C-terminal PDZ domain (PDZ). Arrowheads indicate location of tyrosine residues in relation to the functional domains of Yap.
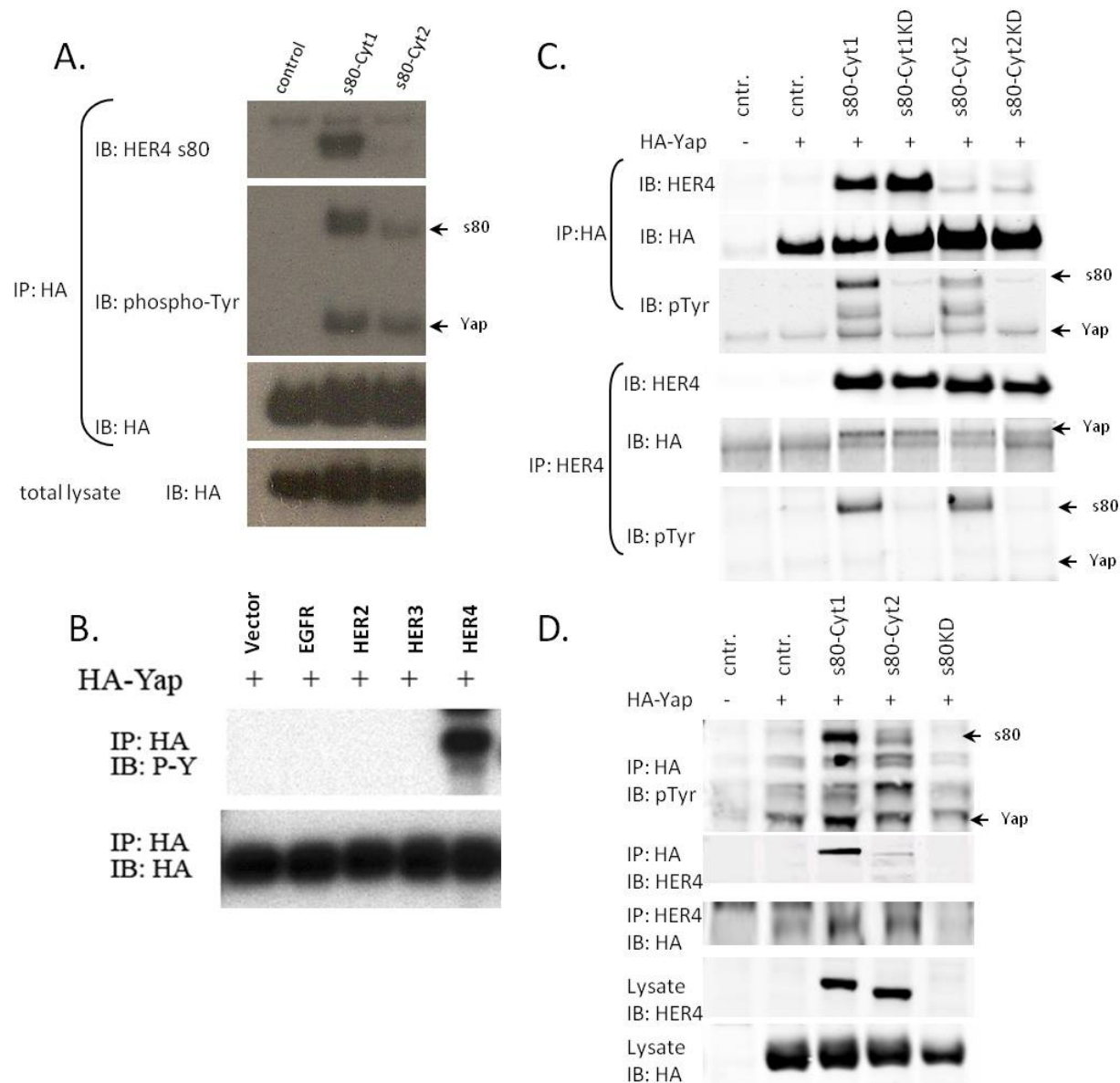
Figure 3. Yap preferentially binds HER4-s80-Cyt1. A. Preliminary data indicating that Yap preferentially binds HER4-s80-Cyt1, as compared to –Cyt2. **B.** Yap binding with EGFR family members. EGFR family members were co-expressed with HA-Yap in COS7 cells and 48h later lysates interrogated for receptor-Yap interaction. **C and D.** HER4-s80 interaction with Yap in COS7 (C) and 293T (D) cells. HA-Yap was co-expressed with s80-Cyt1, -Cyt2 or kinase dead (KD) mutants. 48h after transfection cells were lysed and lysates immunoprecipitated and analyzed by immunoblot as indicated.
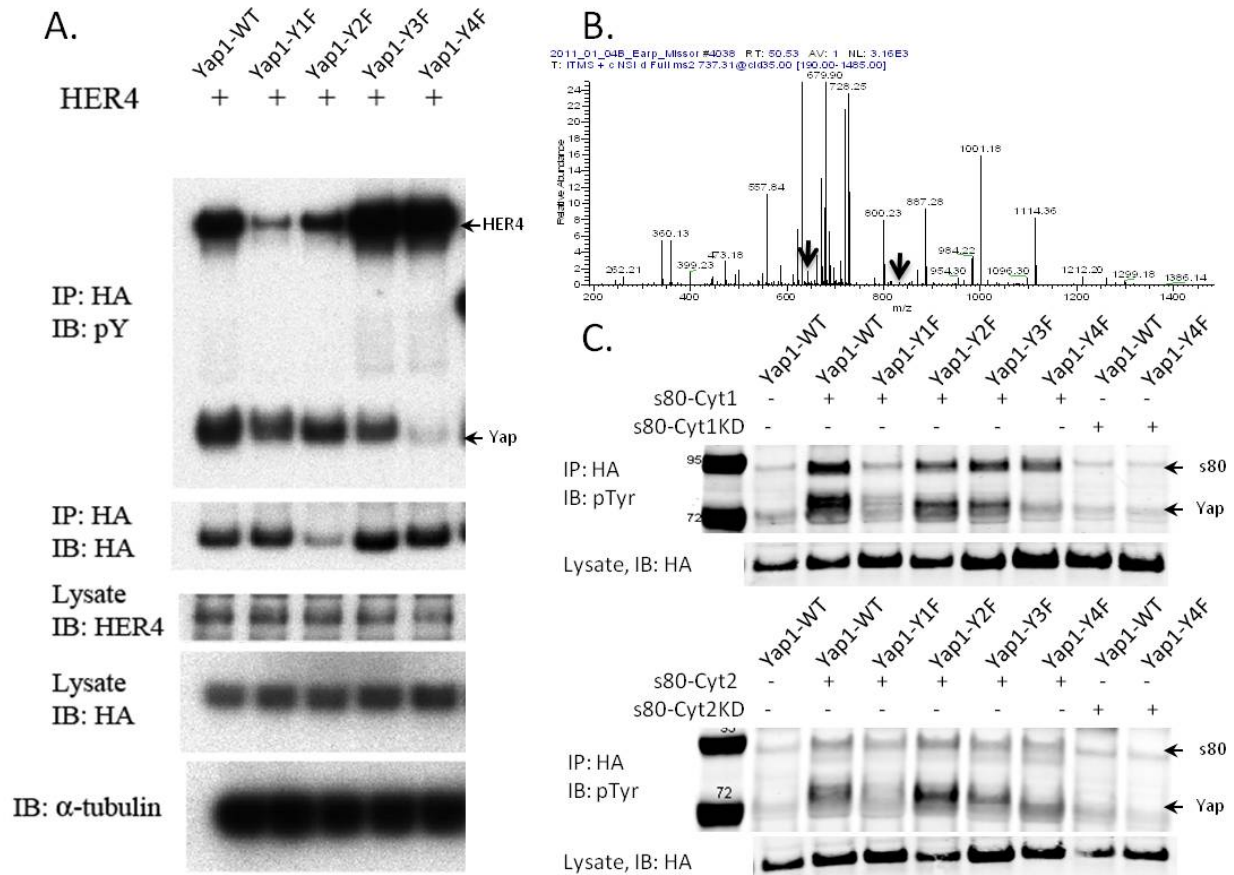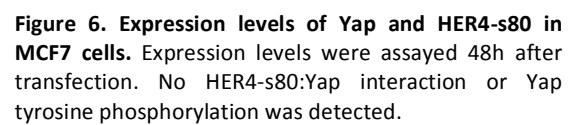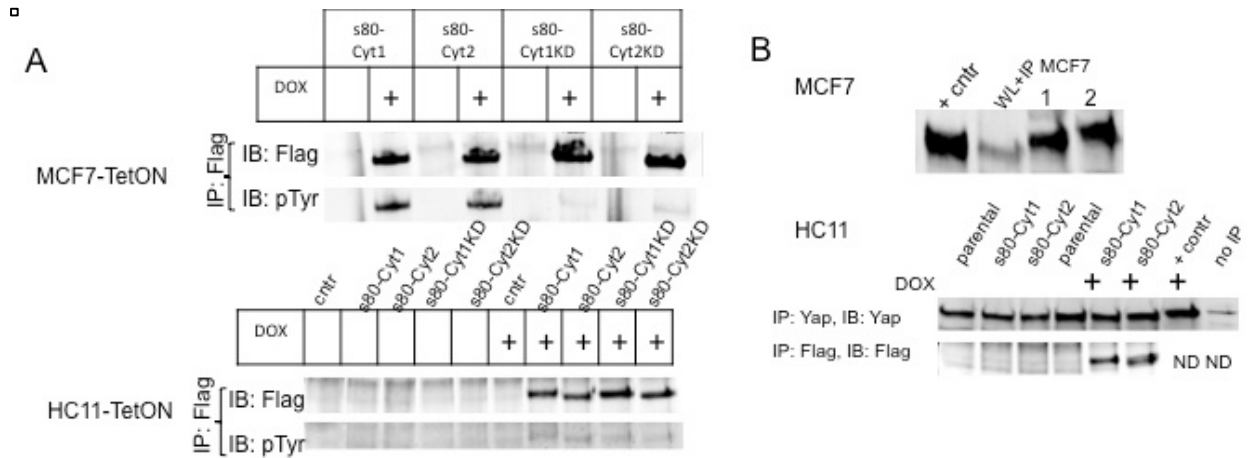
Figure 4. HER4 phosphorylates Yap1 on Y341 and Y394. A. HER4-s80-Cyt1 was co-expressed with HA-Yap WT or indicated mutants in COS7 cells. 48h after transfection lysates were immunoprecipitated as indicated and analyzed by immunoblotting. **B.** Mass spectrum of peptide fragment containing Y394 of Yap1 for manual confirmation of phosphorylation. HA-Yap (WT) was co-expressed with HER4-s80-Cyt1 in COS7 cells. 48h later cells were lysed, lysate immunoprecipitated with anti-HA affinity gel, and run on polyacrylamide gel. Band corresponding to HA-Yap (~72kDa), was excised and digested with AspN, then analyzed on LTQ Orbitrap mass spectrometer. A total of 11 spectra matched to peptides containing Y394 and two were potentially phosphorylated. Manual verification of these peptides supports the phosphorylation of Y394. Arrows indicate peaks used to distinguish pTyr vs. pThr. Study completed with help from the University of North Carolina Proteomics Core Facility. **C.** Comparison of Yap phosphorylation by Cyt1 and Cyt2. COS7 cells were co-transfected with control vector (pcDNA3.1), s80-Cyt1, -Cyt2 or kinase dead variants and either wild type or mutated HA-tagged Yap. 48h after transfection, cells were lysed, lysates immunoprecipitated with anti-HA affinity gel, and analyzed by immunoblotting as indicated.

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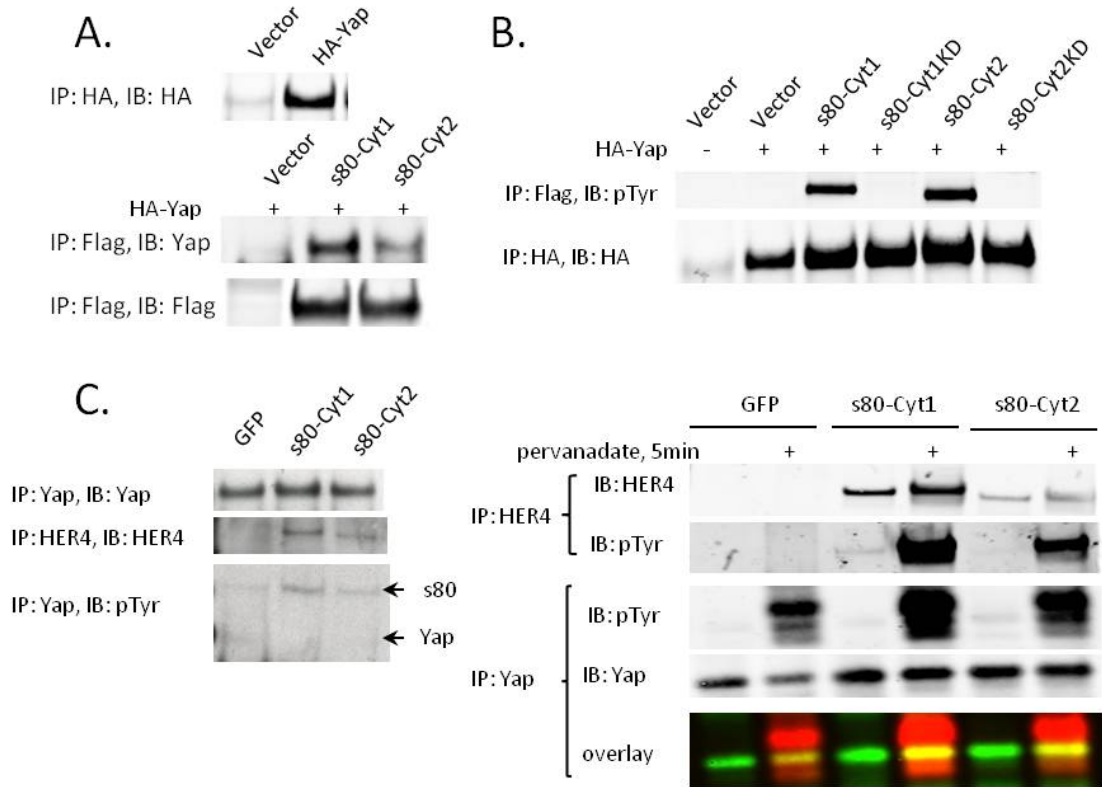
Figure 5. Interaction of HER4-s80 and Yap in mouse mammary epithelial cells, HC11. A. Levels of HER4 isoforms and Yap 48h after electroporation with Amaxa system. Yap binding to HER4-s80 was detected, however not tyrosine phosphorylation of Yap. B. Levels of HER4 isoforms and Yap 48h after reverse transfection. No HER4:Yap interaction, nor Yap tyrosine phosphorylation were detected. C. Levels of HER4-s80 expression and endogenous Yap in HC11 cells stably expressing GFP, GFP-s80Cyt1 or GFP-s80Cyt2 (after retroviral infection). Left panel: Cyt1 and Cyt2 were detected on immunoblots of cell lysates immunoprecipitated with anti-Yap antibody. Right panel: cells were treated for 5 min prior to harvest with vehicle or pervanadate. Tyrosine phosphorylation of Yap and HER4-s80 is only detected in presence of pervanadate. Overlay panel shows co-localization of the pTyr and total Yap signals (detected by Li-Cor detection system (Odyssey)).

Figure 6. Expression levels of Yap and HER4-s80 in MCF7 cells. Expression levels were assayed 48h after transfection. No HER4-s80:Yap interaction or Yap tyrosine phosphorylation was detected.

Figure 7. Inability to detect interaction of HER4-s80 and Yap in mammary epithelial cells. A. Expression of Flag-tagged HER4-s80 constructs (Cyt1, Cyt2, kinase dead Cyt1 (1KD) and Cyt2 (2KD)) in MCF7 and HC11 TetOn cells is detectable only after exposure of cells to doxycycline (here for 24h). B. Detection of endogenous Yap in MCF7 and HC11 cells. Despite adequate expression of endogenous Yap and HER4-s80, the interaction of the two proteins was not detected by co-immunoprecipitation (data not shown).

Panel 1.
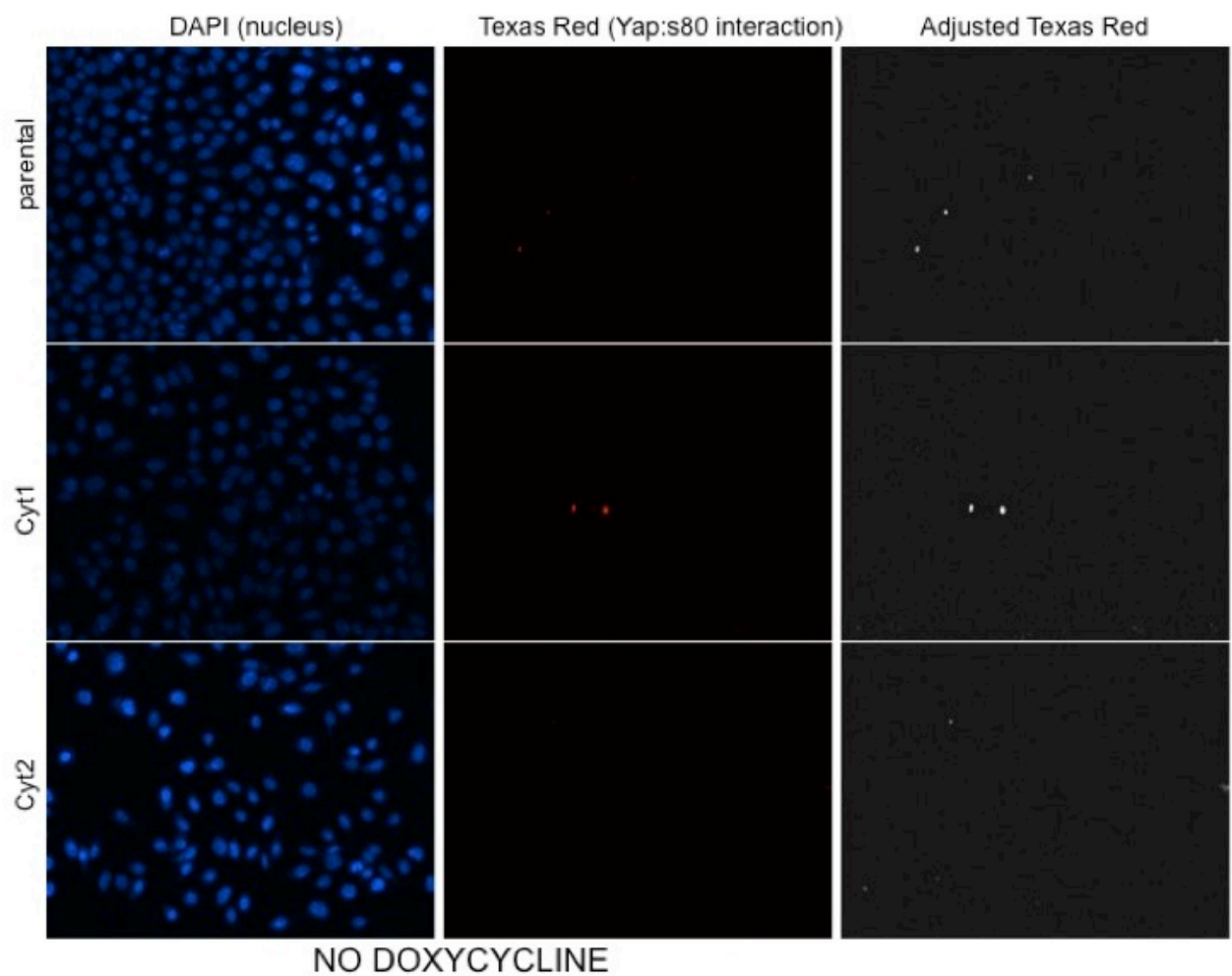
Figure 8. Inability to detect interaction of HER4-s80 and Yap in mammary epithelial cells. Proximity ligation assay in HC11 TetOn cells grown in absence (Panel 1) or presence (Panel 2) of doxycycline for 24h. Nuclei were detected by staining with DAPI, positive interaction of HER4-s80 and Yap was detected in Texas Red channel. (Third column contains enhanced black and white images for better evaluation of data in printed form).

Panel 2.

Adjusted Texas Red

Texas Red (Yap:s80 interaction)

DAPI (nucleus)

parental

Cyt1

Cyt2

WITH DOXYCYCLINE

32
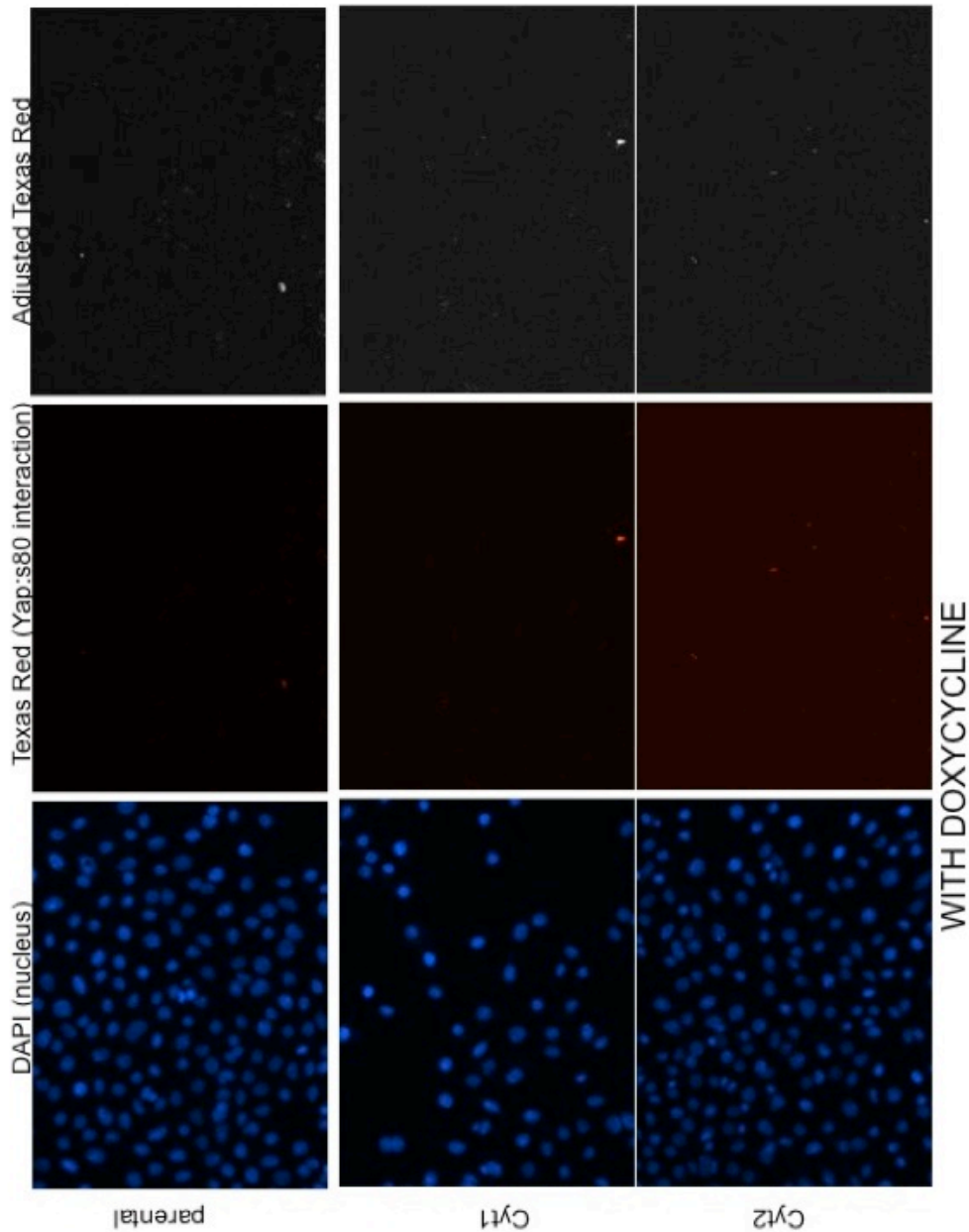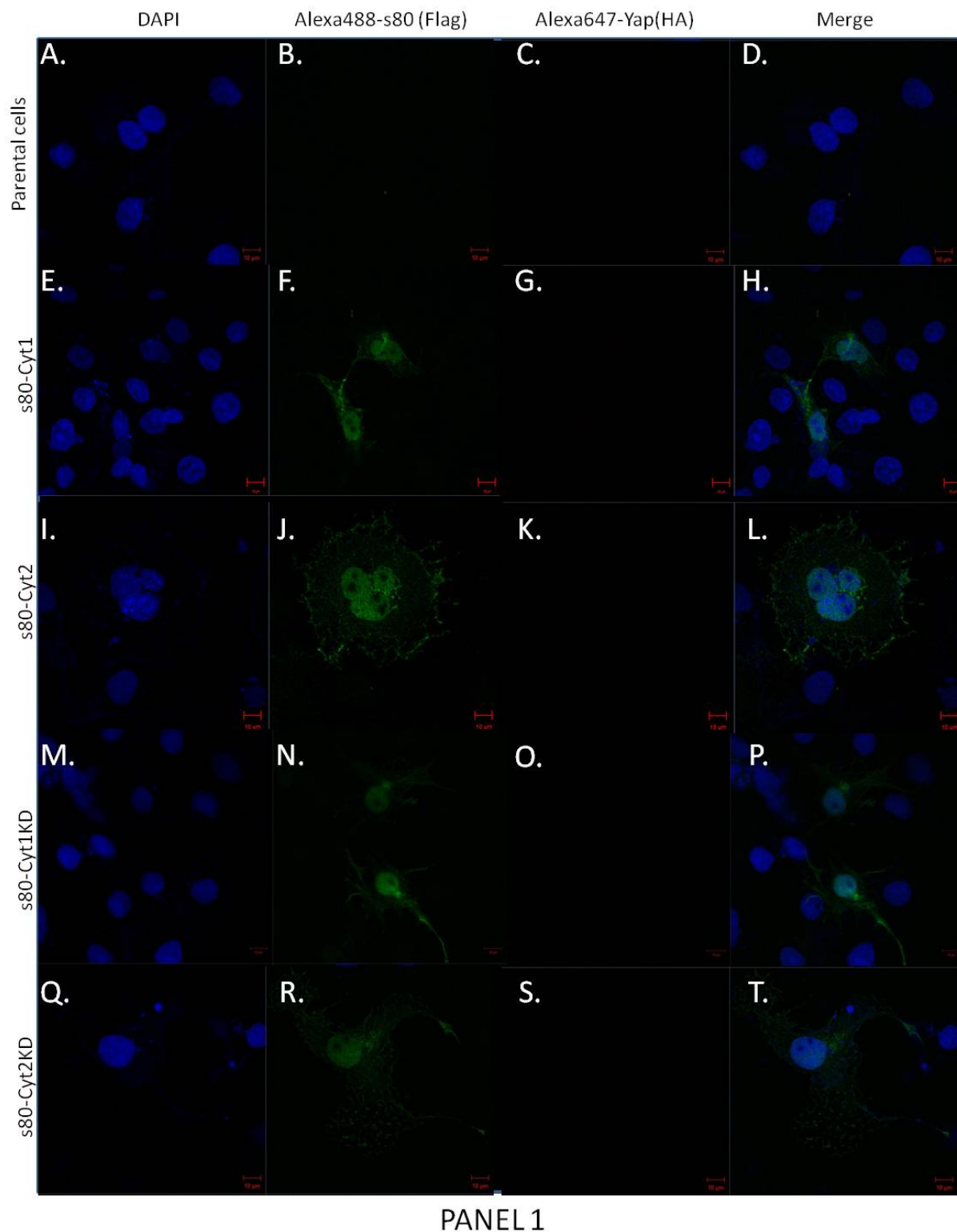
Figure 9. Localization of Yap and HER4-s80 isoforms in COS7 cells. COS7 cells were transfected with indicated constructs and 24h later plated on slides. After 24h to allow for cell attachment and recovery, cells were fixed, permeabilized and stained with anti-HA antibody coupled to Alexa647 and anti-Flag antibody coupled to Alexa488 (Cell Signaling). Slides were mounted with mounting media for fluorescence containing DAPI and analyzed with Zeiss LSM710 confocal microscope. All photographs taken at 63x with oil immersion. Scale bars represent 10 µm.

DAPI	Alexa488-s80 (Flag)	Alexa647-Yap(HA)	Merge

Yap

A. B. C. D.

Yap+Cyt1

E. F. G. H.

Yap+Cyt2

I. J. K. L.

Yap+Cyt1KD

M. N. O. P.

Yap+Cyt2KD

Q. R. S. T.

PANEL 2
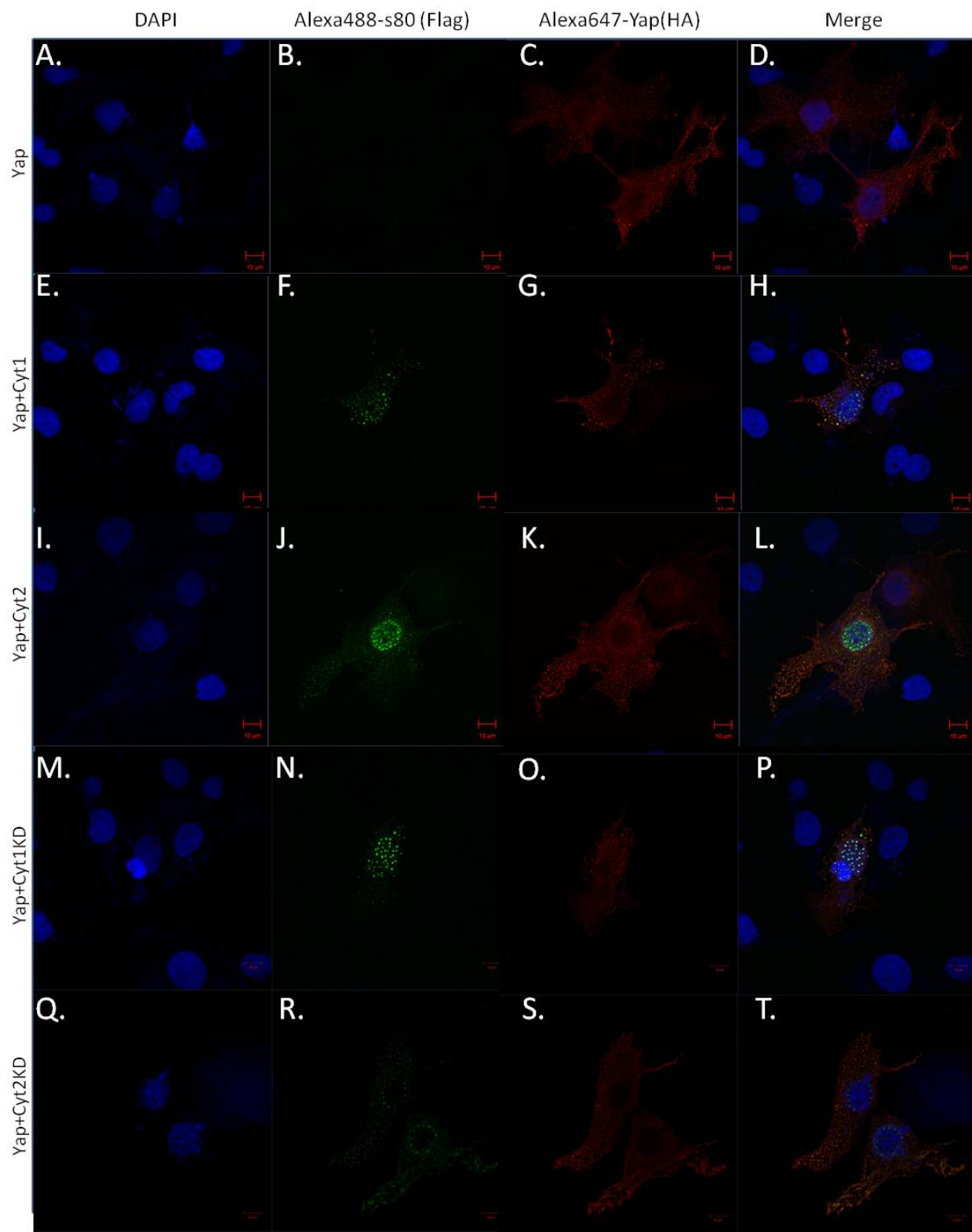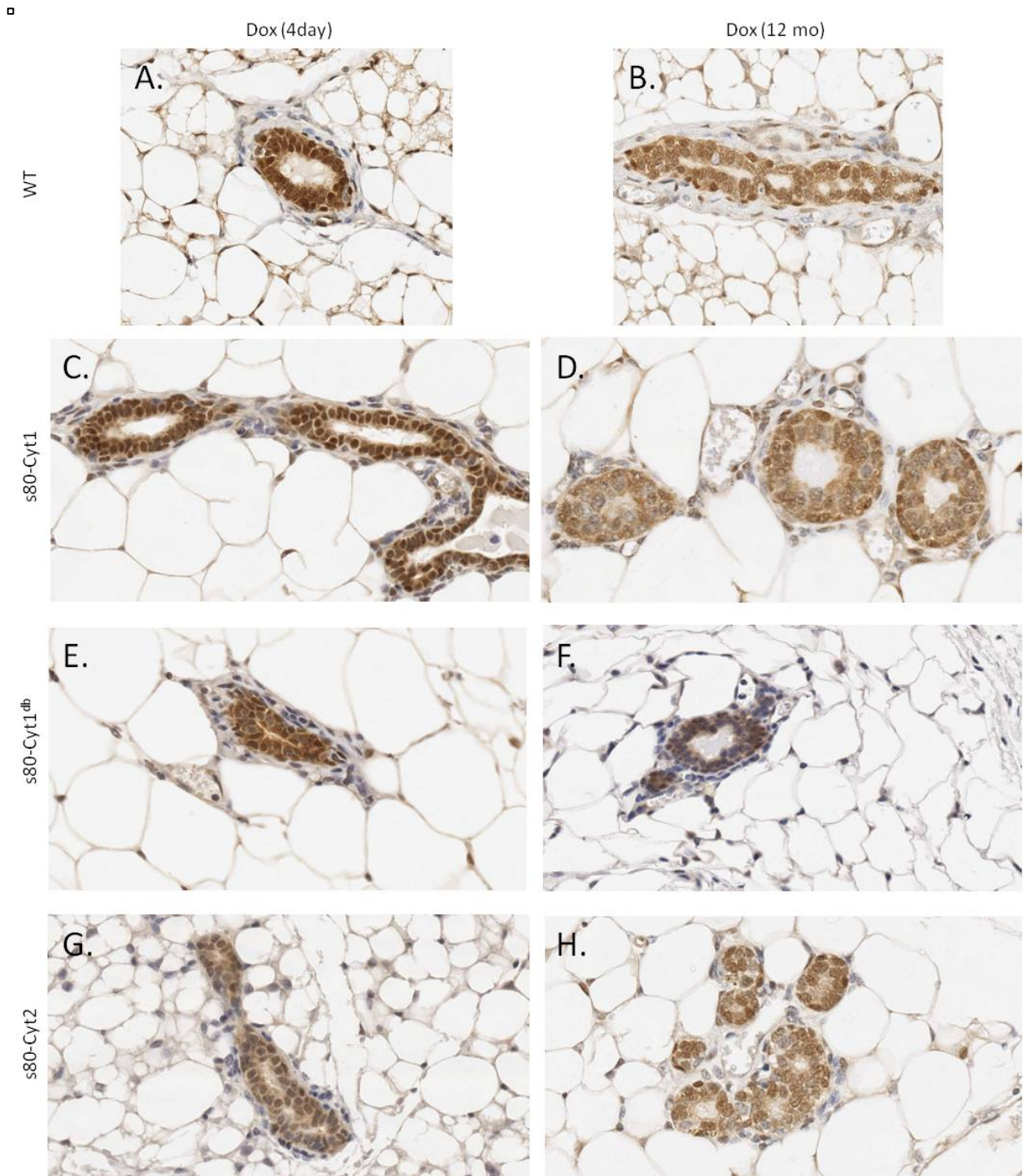
Figure 10. Localization of Yap in murine mammary epithelial cells. Mammary glands were harvested from WT and transgenic mice expressing human HER4-s80-Cyt1, -Cyt2 or degradation resistant –Cyt1[db] for either 4 days or 12 months. Tissue was fixed in formalin and embedded in paraffin then stained with Yap antibody following an established immunohistochemistry protocol. Sections were counterstained with hematoxylin. Slides were photographed at 40x.
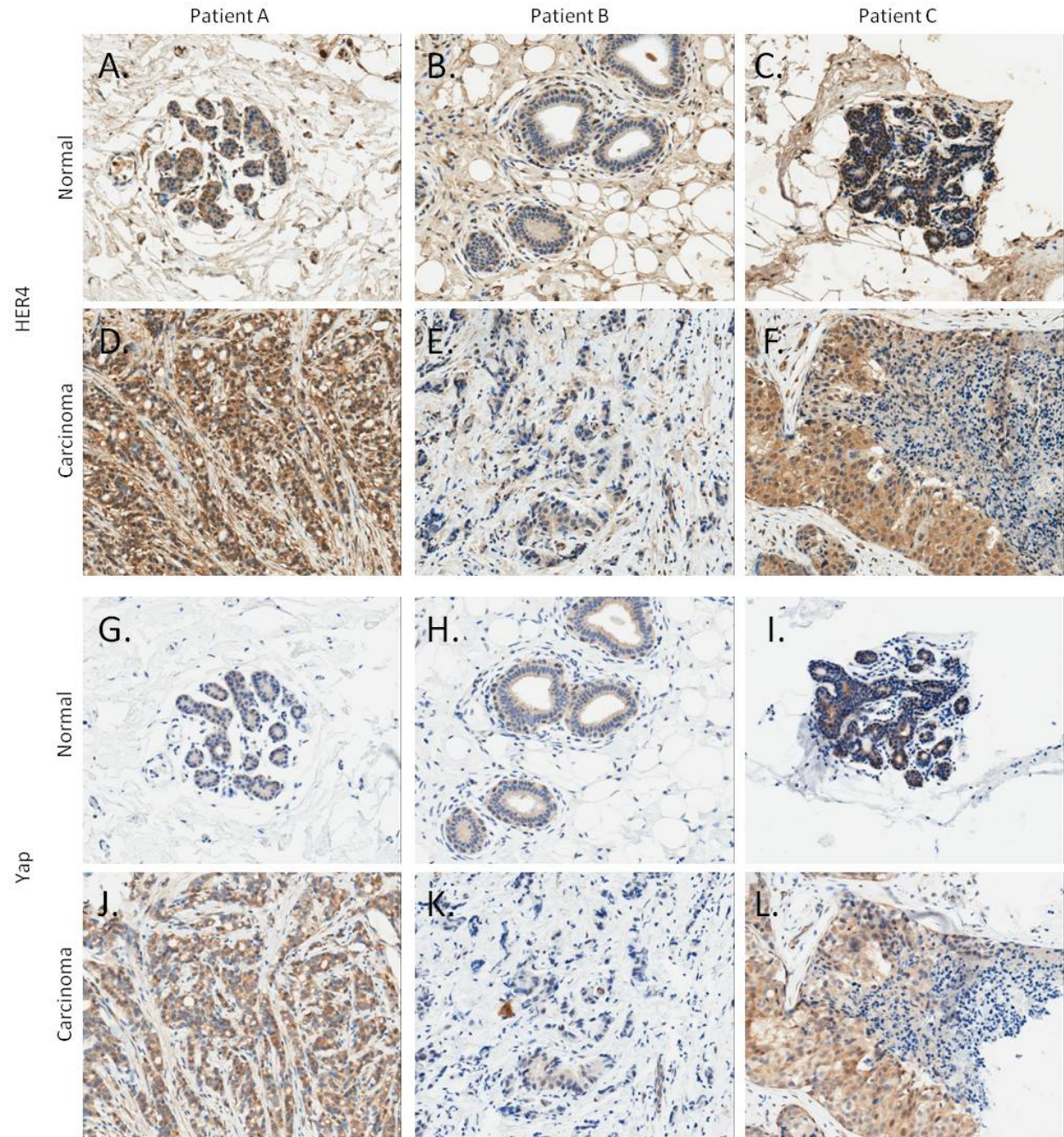
Figure 11. Yap and HER4 expression in human breast carcinoma and matching normal tissue. 10 samples of estrogen receptor-positive breast carcinoma and matching normal tissue were obtained from the University of North Carolina Tissue Procurement Facility. Sections were stained for Yap and HER4 following established immunohistochemistry protocol. Slides were scanned and scored for cytoplasmic and nuclear localization. Panels A-F: HER4 staining in normal (A-C) and carcinoma (D-F) samples from three patients. Panels G-L: Yap staining in normal (G-I) and carcinoma (J-L) samples from three patients. All images acquired at 20x.
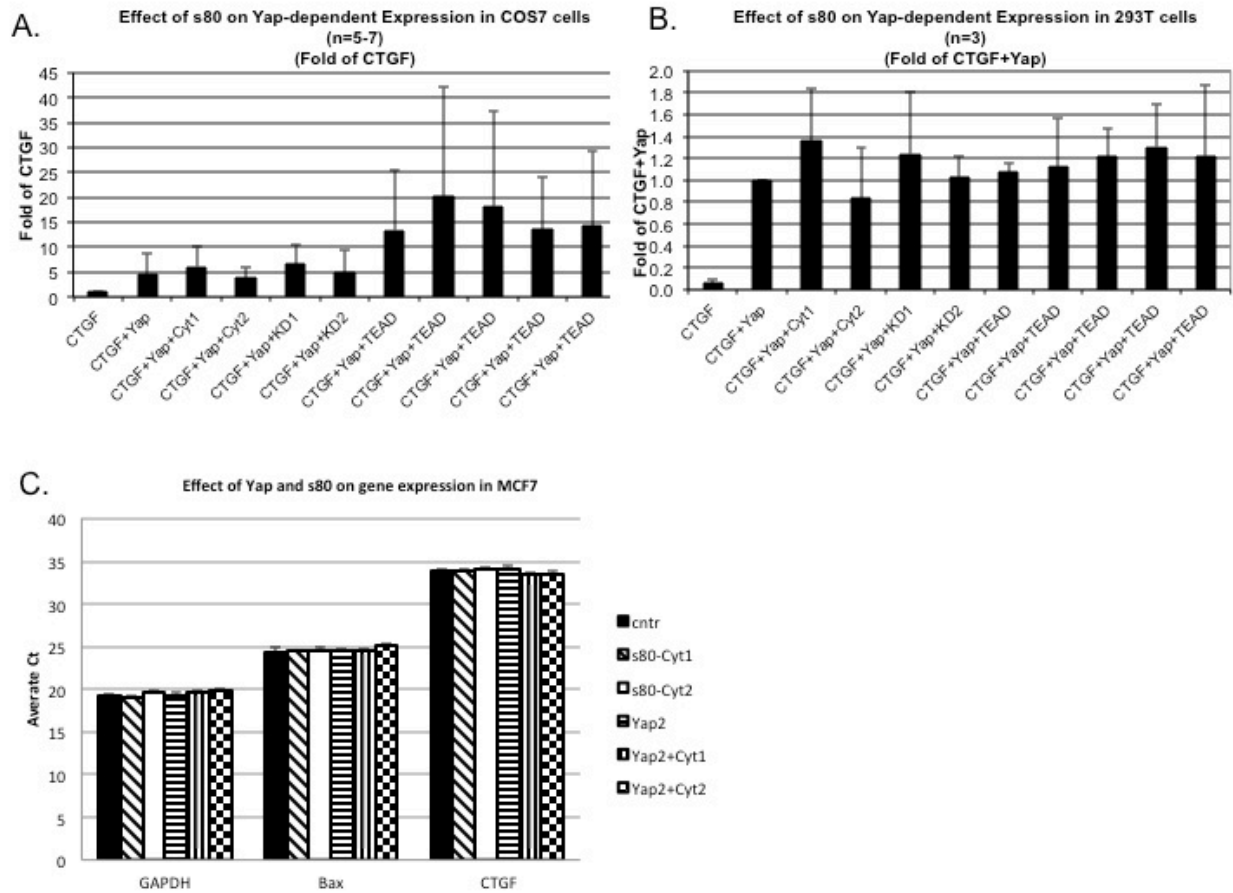
Figure 12. HER4 effects on Yap-dependent transcription. Expression of luciferase reporter driven by CTGF-promoter in 293T (A) or COS7 (B) cells. Cells were transfected with the reporter and indicated constructs and luciferase expression was measured 48h later. Cumulative data from 5-7 (A) and 3 (B) experiments, respectively. C. MCF7 cells were transfected with indicated constructs and expression of GAPDH (control), Bax and CTGF measured 48h later by real time PCR.

A.

	cntr	Cyt1	Cyt2	Yap WT	Yap Y4F	Cyt1		Cyt2	
						Yap WT	Yap Y4F	Yap WT	Yap Y4F

IP: Flag, IB: Flag

IP: HA, IB: HA

B. Effect of Yap phosphorylation on Yap-dependent trancriptional activity
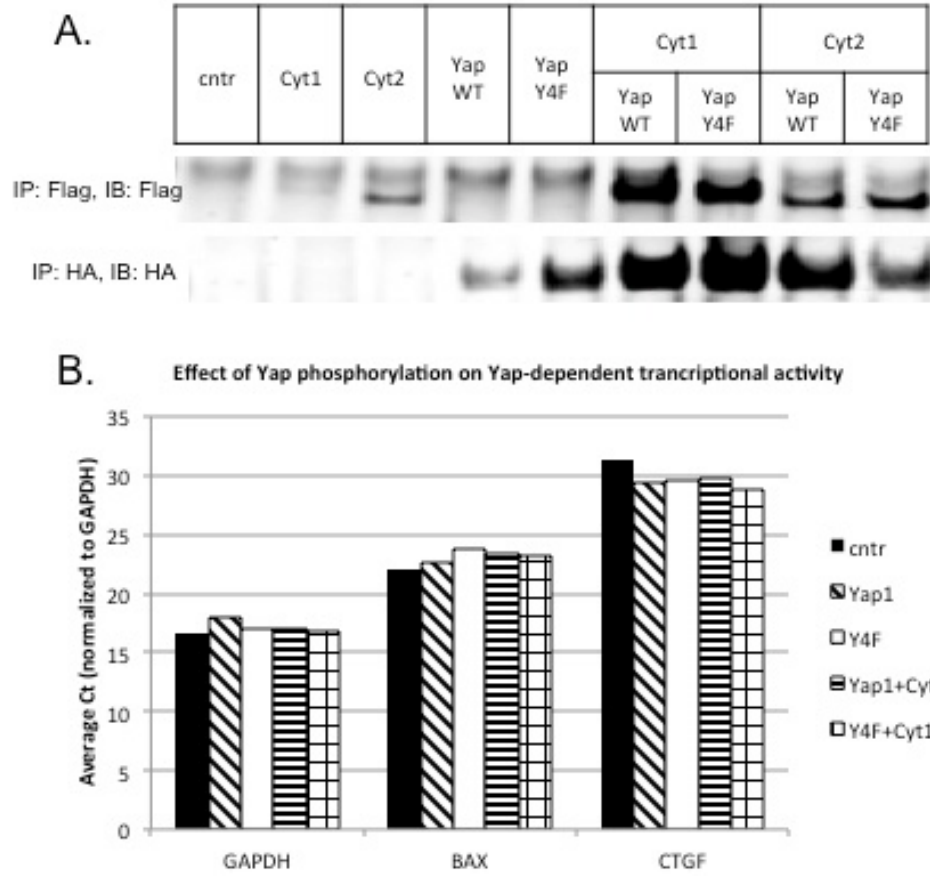
Figure 13. Effect of Yap phosphorylation on Yap-dependent transcription. MCF7 mammary epithelial cells were transfected with indicated constructs and cells were harvested 48h later. Cell lysates were used for immunoblottoing to evaluate construct expression (A), and total RNA was isolated and used for real time PCR to evaluate expression of GAPDH (control) and Yap-regulated genes (Bax and CTGF; B).

A.

TEAD4-Myc		+	+	+	+	+	+	+	+	+
HA-Yap2							+	+	+	+
Flag-Cyt1			+				+			
Flag-Cyt1KD				+				+		
Flag-Cyt2					+				+	
Flag-Cyt2KD						+				+

IP: Myc
IB: pTyr → HER4 (95), → Yap (72), → TEAD (55)
IB: HER4
IB: Myc

IP: Flag
IB: Flag
IB: Myc

Lysate
IB: HA
IB: Flag
IB: Myc
IB: tubulin

B.

Contr.	+	+	+		+	+	+		+	+	+	
Flag-s8D-Cyt1		+		+		+		+		+		+
Myc-TEAD			+	+		+	+			+	+	
MG132					+	+	+	+				
Bafilomycin A1									+	+	+	+

IB: Flag — 55.88 39.26 61.59 35.27 42.70 27.78
IB: Myc — 61.16 59.62 68.41 61.49 60.30 48.86
IB: tubulin
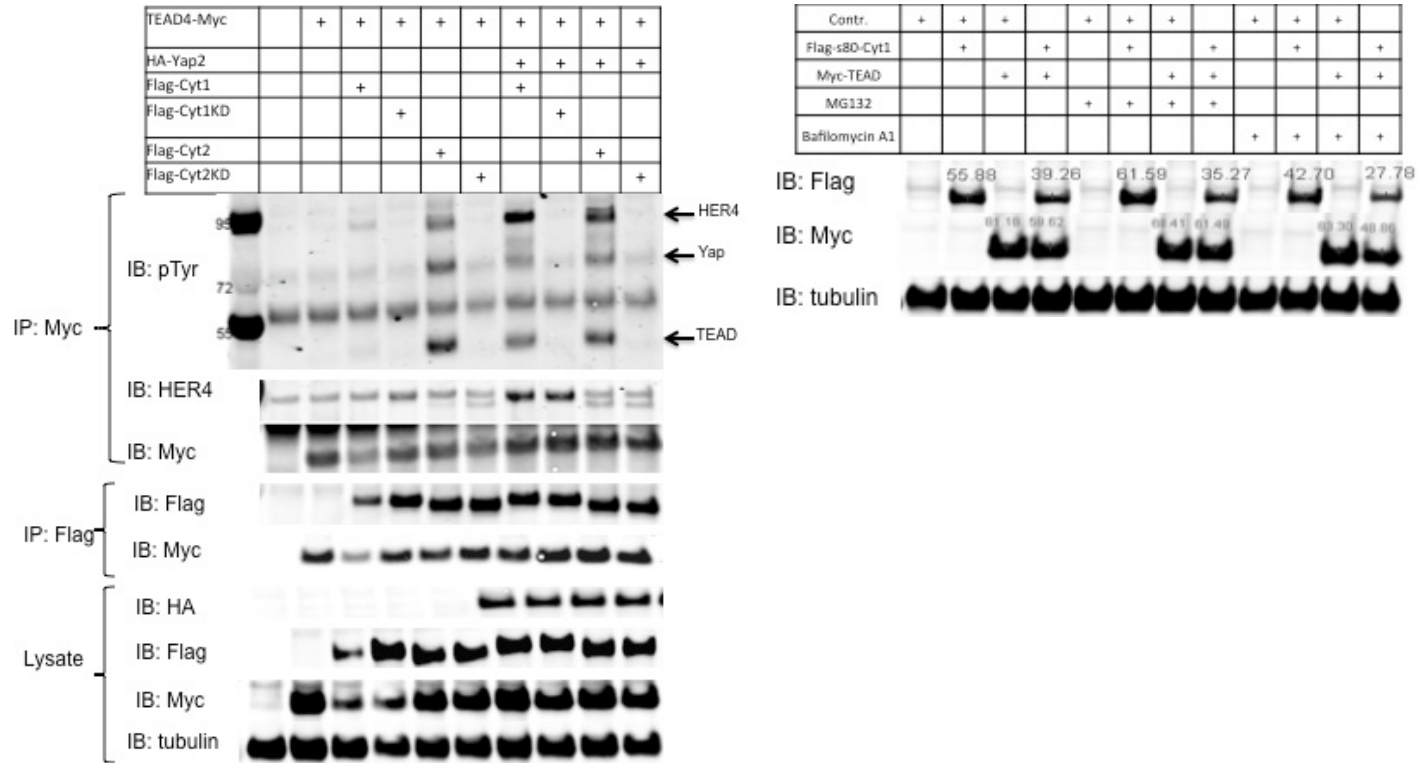
Figure 14. Interaction of HER4 with TEAD/TEF: Yap complex. A. COS7 cells were transfected with indicated constructs and lysates generated 48h later, then immunoprecipitated with anti-Myc beads to enrich for TEAD-associated complexes. Protein tyrosine phosphorylation, as well as protein expression was detected by immunoblotting. B. Effect of MG132 (proteasome inhibitor) and Baflilomycin A1 (lysosome inhibitor) on Cyt1 and TEAD/TEF expression in COS7 cells. Cells were transfected as above and incubated with inhibitors prior to cell lysate harvest. Numbers above bands indicate raw band density as measured by the LiCor Odyssey Imaging System.

Sequence	Modifications	Exp Value	Charge	First Scan	Last Scan	Ascore	Site Determining Ions	Localized
DSFFKPPEPKsHSRQAST	S11(Phosp)	0.001972287	3	4001	4001	5.99	1 / 22 @ 1 pks	no
DPFLNSGTyHSR	Y9(Phosp)	0.000189657	2	4038	4038	20.35	2 / 2 @ 8 pks	Yes
DPFLNSGTyHSR	Y9(Phosp)	0.001534511	3	4040	4040	6.93	2 / 8 @ 5 pks	no
DSFFKPPEPKSHSRQAsT	S17(Phosp)	0.000370655	3	4074	4074	0.06	0 / 1 @ 10 pks	no
DPFLNSGTYHsR	S11(Phosp)	0.000306881	2	4544	4544	57.75	4 / 4 @ 3 pks	Yes
DLGtLEG	T4(Phosp)	0.002079553	1	4551	4551	Only 1 site		
DDFLNsV	S6(Phosp)	0.001636703	1	6721	6721	Only 1 site		
DFLNsV	S5(Phosp)	0.00064561	1	6884	6884	Only 1 site		
DyLEAIPGTNV	Y2(Phosp)	0.000120773	1	7215	7215	0	0 / 0 @ 5 pks	no

Table 1. Mass spectrometry analysis of Yap1 phosphorylation sites. Yap1 was co-expressed with HER4-s80-Cyt1 in COS7 cells, lysate harvested 48h later and immunoprecipitated for HA-Yap and run on polyacrylamide gel. Band corresponding to HA-Yap (~72kDa), was excised and digested with AspN, then analyzed on LTQ Orbitrap mass spectrometer. ASCORE > 20 implies localization of phosphorylation on peptide [35]. These results were confirmed manually for peptide containing Y341 and Y394.

	Short Term (4 day)	Long Term (one year)
WT	5	4
s80-Cyt1[db]	3	3
s80-Cyt1	9	5
s80-Cyt2	7	3

Table 2. Yap localization in murine mammary gland. Numbers of mice per each group for the analysis of Yap localization in mammary glands of transgenic mice expressing human HER4-s80-Cyt1, -Cyt1[db] or –Cyt2.